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<p>(54) Title: IDENTIFYING BIOLOGICALLY ACTIVE AGENTS THROUGH CULTURE COLOR CHANGE (57) Abstract <p>The present invention relates to a method of identifying a biologically active agent, which affects a targeted cellular component, whose existence or function is essential for cell viability, pathogenesis or drug resistance. This assay method includes the use of a mixed culture of first and second cell strains. The cell strains may be either strains of different cells or different strains of the same cell type. The two cell strains are distinguished by color differences. The first cell strain has a first color while the second cell strain displays a second color. The second cell strain is also more sensitive towards a class of biologically active agents than is the first cell strain. The first and second cell strains, and a candidate agent are mixed with a medium, appropriate for growth of the strains, forming a mixed assay culture. The culture is then exposed to conditions appropriate for growth. The initial proportions of the first and second cell strains are such that the culture predominantly favors the development of the second color. The biological activity of the agent is then determined from the color of the culture. Where the mixed culture retains the second color, the agent was not active against the target. However, where the culture predominantly becomes the first color, then the agent showed specific activity towards the target.</p></p>		

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IDENTIFYING BIOLOGICALLY ACTIVE AGENTS THROUGH
CULTURE COLOR CHANGE

Background of the Invention

- Assays for detecting biologically active agents should possess the attributes of simplicity, sensitivity and specificity. A. Imada and K. Hotta, Historical Perspectives of Approaches to Antibiotics Discovery, Emerging Targets in Antibacterial and Antifungal Chemotherapy, Chapman and Hall (1992).
- Simplicity is needed as thousands of samples may be tested. Assay sensitivity is needed to evaluate low concentration active compounds derived from various sources, such as fermentation broths or plant extracts. Specificity is important to identify compounds that are active against a specific target, such as a gene product, a cellular structure, or a component whose function is needed for pathogenesis or drug resistance.
- In current methods of assaying for biologically active agents, an agent to be assessed for a desired activity is contacted with a pure culture of a single cell strain. In these methods, it is often difficult to readily assess the comparative activities of an agent against different cell strains, since they are cultured separately. This comparison is typically needed to identify agents with target specific activity. This is particularly difficult for those fungi cultures, which grow with clumping where growth is difficult to measure on a routine basis.
- Therefore, a need exists for a simple assay method for comparing the relative activity of an agent against a specific target in different cell strains in a single test, rather than in multiple tests.

-2-

Summary of the Invention

The present invention relates to a method of identifying a biologically active agent which affects a targeted cellular component. This method makes use of a mixed culture of two cell strains, which may be either strains of different cells or different strains of the same cell type. The cell strains in the mixed culture are distinguishable from one another on the basis of a phenotypic difference and exhibit different responses to an agent, referred to as a candidate agent. The first cell strain of the mixed culture, referred to as the control strain, displays a first color. The second cell strain of the mixed culture, referred to as the test strain, displays a second color and is more sensitive, than the first strain, to a biologically active agent which is active against the targeted cellular component.

The test and control strains used can be naturally occurring (wild type) strains which differ on the basis of color and sensitivity to a biologically active agent or can be mutated or genetically engineered cell strains, in which an endogenous gene (or genes) has been altered to produce the phenotypic differences (color and/or drug sensitivity) or which have been altered by the introduction of an exogenous gene (or genes) whose presence results in the phenotypic differences.

In the present method, referred to as a mixed culture assay, a suitable ratio of the first and second cell strains, and an agent to be assessed for its biological activity (a candidate agent) are mixed with

-3-

medium which is appropriate for growth of the strains, thereby forming a mixed culture wherein the ratio of cell strains in the mixed culture predominantly favors the development of the test strain color. This mixed culture is then exposed for a suitable period of time to conditions appropriate for culture growth (growth of the cells). The biological activity of the agent is then evaluated by determining the color of the mixed culture. If the mixed culture retains a mixture of the colors, or is the second color, the candidate agent is not biologically active. However, where the mixed culture takes on the first color, the candidate agent has demonstrated specific biological activity towards the targeted cellular component and is a biologically active agent.

This invention has the advantage that it demonstrates assay simplicity by reducing the number of tests, which must be performed to evaluate a candidate biologically active agent. This assay also permits the comparison of the relative activities of an agent against different cell strains. Thus, this comparison permits better identification of target-specific biological agents.

Brief Description of the Drawings

Figure 1 is a schematic representation of an assay of a biologically active agent of the present method using a strain of Saccharomyces cerevisiae expressing a gene of Candida albicans which confers on Saccharomyces cerevisiae multiple drug resistance.

-4-

Figure 2 is a schematic representation of an integrative plasmid of Saccharomyces cerevisiae which contains a multiple drug resistant gene of Candida albicans.

5 Figures 3 and 4 are graphical representations of the virulence of a wild type and mutant strains of Candida albicans ATCC 10261 against male ICR mice.

10 Figure 5 is a graphical representation of the virulence of csd2/csd2 and csd2/CSD2 mutant strains of Candida albicans against male ICR mice.

Detailed Description of the Invention

The present invention is a method of identifying a substance, referred to as a biologically active agent, which has a desired activity or activities against a
15 targeted cellular component, such as anticancer agent activity, anti-fungal activity, anti-bacterial activity or activity towards other organisms, such as weeds or insects. As defined herein, a targeted cellular
20 component is any cellular component, such as a gene product, enzyme, cell structure, etc., whose existence or function is essential for cell viability or is needed for pathogenesis or drug resistance.

In the present method, a suitable mixed culture of cell strains is contacted with a substance to be
25 assayed for its biological activity. Cell strains comprise either strains of different cell types or different strains of a particular cell type.
Furthermore, as defined herein, a mixed culture is a culture containing a mixture of at least two cell
30 strains, wherein said cell strains have different

-5-

levels of susceptibility to a class of biologically active agents towards the targeted cellular component. For example, one cell strain may carry a mutation in a gene encoding a target protein. A mutated cell strain, as defined herein, is a cell strain which is modified such that it includes at least one gene, wherein said modification changes the color of the mutated cell strain and/or increases or reduces the sensitivity of the mutated cell strain to a biologically active agent. The modification can be an alteration (disruption, removal, turning on, enhancement of expression) of an endogenous gene(s) or introduction of an exogenous gene (or genes)

In addition, each cell strain contained in the mixed culture possesses a discernible phenotypic characteristic, such as color, that is different from the characteristic of the other cell strain in the culture. Changes in phenotypic characteristic of the mixed culture, or lack of such changes, after culturing with a candidate agent, indicate the biological activity of the agent against the targeted cellular component.

In one embodiment of this method, a mixed culture comprises a mixture of two or more strains of a cell, wherein at least one strain is a control strain and at least one strain is a test strain. Suitable cells include any cultivated cell type displaying a distinct color, for example, Saccharomyces (S.) cerevisiae, Aspergillus (A.) nidulans (also known as Emmericella nidulans), Candida (C.) albicans, bacteria, insect cell lines, mammalian cell lines and plant cells.

-6-

The control strain, as defined herein, has a lower sensitivity towards the class of agents, which are biologically activity towards a targeted cellular component, while the test strain has a greater
5 sensitivity towards the same class of agents.

In another embodiment, the test strain and control strain include a different allele of a specific gene which encodes a product required for cell growth, reproduction, survival or development, wherein a
10 candidate biologically active agent is targeted against a cellular component which includes said gene product.

The control strain and the test strain must also be distinguishable on the basis of a difference in a phenotypic characteristic, such as color. Changes in
15 the phenotypic characteristic after the mixed culture is contacted with a candidate agent, are due to a change in the ratio of the two strains in the mixed culture. In another embodiment where color is the phenotypic characteristic, the test strain possesses a
20 color resulting from a wildtype gene or from a mutant gene, and the control strain either possesses its own (normally occurring) color or has been modified (e.g., by mutation of an endogenous gene or introduction of a gene which confers color) to obtain a distinct
25 coloration. The color of the test strain is a first color. The color of the control strain is a second color, which is different from the first color.

In this mixed culture, prior to being contacted with a candidate biologically active agent, the second
30 color predominates because the ratio of cell types is adjusted in favor of the color attributable to the test

-7-

strain. After the mixed culture has been contacted with the substance to be assayed, and the resulting mixed culture has been maintained for an appropriate incubation period, the second color continues to dominate the mixed culture, the mixed culture develops the first color, or the mixed culture has neither the first color nor the second color.

Where the mixed culture, which has been contacted with a candidate agent, continues to exhibit the mixture of the two colors or the second color, then the candidate agent in the amount tested is not substantially biologically active against the targeted cellular component (i.e., is not a biologically active agent). If the mixed culture, which has been contacted with a candidate agent for an appropriate period of time, exhibits the first color, it is evidence that the candidate agent, in the amount tested, demonstrated specific biological activity against the targeted cellular component (i.e., is a biologically active agent). This activity substantially inhibits the growth and/or development of the test strain allowing development of the first color, due to growth of the control strain. Furthermore, if a culture which has been contacted with a candidate agent lacks both the first color and the second color, it is indicative that the substance, in the amount tested, was generally active against or toxic to the cells strains in the mixed culture, thus resulting in the lack of color formation, such as due to the inhibition of cell growth or of cell development, or due to the death of cells of both cell strains.

-8-

In the present method, the control strain and the test strain are grown or cultured together, such as within or on a single culture medium, in the presence of a substance which is a candidate biologically active agent. Thus, it is possible to grow the strains to be compared, together, in order to more readily assess the effects of the candidate biologically active agent, and to assess the effects on the basis of a single color determination.

It is to be understood that the control strain, the test strain and the candidate agent can be introduced into or onto the surface of the culture medium concurrently or in any desired order. Further, the candidate agent can be introduced into the medium either individually or in a mixture, such as a broth.

To determine the cell strain which is a suitable test strain, an appropriate cell strain sensitivity test is conducted. Such sensitivity tests are known in the art and include, for example, sensitivity tests wherein the growth of various cell strains is evaluated in a media containing a known biologically activity agent. Such sensitivity tests are described in Examples 1 and 3.

In addition, to construct an appropriate assay, a suitable ratio of the control strain to the test strain must be established. A suitable ratio is one which makes it possible to determine the relative extent of growth of the two strains. To determine the optimum ratio of the control strain and the test strain, the two strains are mixed in various proportions and grown in the presence or absence of a known biologically

-9-

active agent. The phenotypic characteristic of the culture is then monitored. The optimum ratio of the control and test strains is that ratio, where in the absence of a biologically active agent, the color developed by the mixed culture would be substantially the color of the test strain, but in the presence of a biologically active agent, the color developed by the mixed culture would be substantially the color of the control strain. The correct ratio for a particular pair or combination of more than two test and control strains can be determined as described herein and will differ based on the strains used. The determination of optimum ratios is described in Examples 1 and 3.

The mixed culture assay method of this invention is useful to identify various biological activities of different substances. The potential biological activities that can be evaluated in the mixed culture assay, include inhibition of fungal or bacterial reproduction, fungal or bacterial death, enhancement of DNA repair or DNA binding, and immunosuppression.

In one embodiment of the present method, a mixed culture which is a mutant strain and a wild type strain of a fungus, such as S. cerevisiae or A. nidulans, is used to assay potential anti-fungal agents or, more specifically, potential microtubule disrupting agents. The strains are grown in mixed culture in, or on, media in petri plates or in, or on, media in microtiter tray wells. Candidate biologically active agents (candidate anti-fungal agents) are added, individually or in a mixture, such as a broth.

-10-

Fungi are particularly well suited for use in cell-based assays for compounds or molecules with desired activities, such as drugs useful for preventive, therapeutic or diagnostic purposes. The two species of fungi, Saccharomyces (S.) cerevisiae and Aspergillus (A.) nidulans, are particularly useful in such assays because much is known about their molecular and cellular biology and genetics. Fungi generally can be genetically engineered with a potential drug target from an unlimited number of sources, by introducing into the fungi DNA which encodes a product directly or indirectly related to a target of a biologically active agent. A drug can be a molecule or compound which reduces (totally or partially) or enhances the function or activity (either in terms of type or extent) of the target. Target-specific drugs can be detected and identified, as can drugs which have a general (non-specific) effect. A particular advantage of an assay, in which fungi such as S. cerevisiae or A. nidulans is used, is that sensitive cellular systems are available and are useful to detect target-specific compounds even if they are present at low concentration in the complex chemical mixtures, such as those presented by natural product broths. Some targets are on the surface of microorganisms whereas other targets are internal. Because the assays are cell-based, it is possible to ensure that compounds must cross the cell surface (i.e., are able to cross the cell wall and cell membrane) in order to reach an internal target; those which do not cross the cell surface or do so in inadequate quantities will not be identified as drugs

-11-

which inhibit the internal target. It is to be understood that cells other than S. cerevisiae or A. nidulans can also be genetically engineered to test for a potential drug target.

- 5 In another embodiment, an assay of mixed strains of S. cerevisiae is used to evaluate the biological activity of agents targeted to bypass or inhibit a drug-resistant gene, for example a C. albicans gene which confers resistance to microtubule disrupting
- 10 agents, such as benomyl. In this assay, the sensitivity of the test strain results from the genetic engineering of a strain, which is hypersensitive to a microtubule disrupting agent, to include a drug-resistant gene. The control strain in this assay is a
- 15 cell strain which is less sensitive to microtubule disrupting agents than is the hypersensitive strain. Where the hypersensitive strain does not carry the drug-resistance gene. In one example of this assay, further described in Example 2, the test strain is
- 20 white while the control strain is red due to a mutation in the *ade2* locus. The strains are mixed at a defined ratio so that the colony will appear substantially the color of the test strain. The mixed culture is then contacted with a microtubule disrupting agent, at a
- 25 sublethal concentration, and a candidate anti-drug resistance agent, such as fungal fermentation broths. Where the candidate agent is not biologically active as an anti-drug resistance agent, the mixed culture will remain substantially the color of the test strain.
- 30 However, where the candidate agent has anti-drug resistance activity, the hypersensitivity of the test

-12-

strain to microtubule disrupting agents, will become unmasked and the culture will become the color of the control strain due to the selective killing of the test strain.

5 In yet another embodiment, in which the fungus is S. cerevisiae, the control strain of the mixed culture has a wild type ADE2 locus and is white. The test strain has red pigmentation due to a mutation in the ade2 locus and in addition, carries a mutation carries
10 that increases the sensitivity to chitin synthesis inhibitors. Specifically, this assay uses a mixed culture comprising congenic chs2 ade2 (red) and CHS2 ADE2 (white) strains. In the presence of a candidate agent which is not an active chitin synthesis
15 inhibitor, the mixed assay culture exhibits a pink culture. In the presence of an active chitin synthesis inhibitor, the culture is white because the red chs2 ade2 strain is selectively killed. Candidate agents that are toxins (nonspecific or generally active) will
20 inhibit the growth of both strains, resulting in an absence of pink, white or red color. This mixed culture assay is further described in Example 4.

 In a further embodiment of the present method, wild type and mutant strains of A. nidulans, that each
25 can produce green, yellow, or white conidial spores, are used in an antifungal or microtubule disrupting agent assay. These colors mark a strain with a particular wild type or mutant genotype. As is described in Example 3, the test strain is constructed
30 to carry a mutant or genetically engineered target gene

-13-

that is sensitive to the desired class of biologically active agents, such as microtubule disrupting agents.

In a preferred embodiment, the test strain has white, or green, spores and includes the hypersensitive
5 tubA4 allele, which encodes an abnormal β -tubulin, while the control strain includes the wild type tubA' allele and has green, or white, spores, respectively. When no biologically active agent is added, or an inactive biologically active agent is added, a
10 substantially white (or green) culture develops. However, when a specifically active biologically active agent is added, the white (green) culture is killed or replication-inhibited and a green (white) culture develops. When a generally active biologically active
15 agent is added, both cell strains are killed or inhibited and the culture is neither substantially green or substantially white.

In an alternate embodiment of the present method, a mixed culture assay of cells, such as C. albicans or
20 S. cerevisiae, utilizes a control strain that includes a mutation which reduces the sensitivity of the control strain, toward a class of biologically active agents, as compared to the sensitivity of a strain expressing the wild type allele of the target. The control strain
25 and the test strain are distinguishable on the basis of a difference in a phenotypic characteristic, such as color. Changes in the phenotypic characteristic, monitored after contacting the mixed culture with a candidate agent, are due to a change in the ratio of
30 the two strains in the mixed culture.

-14-

In one embodiment, a mixed culture is used to evaluate candidate agents that bind to cell wall polysaccharides, such as chitin, glucan, and the carbohydrate chains attached to mannan. Suitable binding to cell wall polysaccharides inhibits the growth of fungi. Typical chitin-binding agents include fluorescent dyes, such as Calcofluor, Congo Red, Primulin, and dianethanol. This assay utilizes a mutation which causes a deficiency in the level of cell wall polysaccharides in the strain.

In another embodiment, the mutated control strain resists fluorescent dye binding to chitin due to low chitin levels in the strain resulting from a mutant allele. Thus, in this assay, candidate agents are screened for chitin-binding compounds with antifungal activity. Specifically, candidate agents are screened for compounds that inhibit the growth of wild-type (test) cells but do not inhibit the growth of chitin-deficient (control) cells.

In a preferred embodiment, an alternate mixed culture with congenic *csd2/csd2 ADE2/ADE2* (white, Calcofluor-resistant) and *CSD2/CSD2 ade2/ade2* (pink, Calcofluor-sensitive) strains of *C. albicans*, is used in the assay. To determine the activity of a candidate agent, the color of the culture is monitored. The ratio of red and white strains produces a pink culture in the absence of Calcofluor. In the presence of Calcofluor, the culture is white, since growth of the red (*CSD2/CSD2 ade2/ade2*) strain is selectively inhibited by the Calcofluor. This assay is further described in Example 5.

-15-

Other mixed culture assays for cell wall polysaccharide binding agents employ kre mutants for β -(1,6) glucan-binding assays; kre mutants have reduced levels of β -(1,6) glucan. These mutants are resistant to K1 killer toxin of S. cerevisiae. Similarly, O-linked oligosaccharide-binding assays can employ mutations in a mannosyltransferase (MNT1=KRE2), as these mutations result in the truncation of the O-linked mannosyl oligosaccharides. These mutants are also resistant to K1 killer toxin. Furthermore, for N-linked oligosaccharide-binding assays, the mnm or vrg mutants could be utilized. The vrg mutants are resistant to orthovanadate. Certain mnm mutants are resistant to KT28 killer toxin S. cerevisiae. Additionally, the mnn or vrg mutants may be resistant to pradimicin.

Many other embodiments of mixed culture assays can be utilized to identify biologically active agents. A mixed culture assay can employ a mutant strain of cells, such as S. cerevisiae cells, in which the mutation affects functions required for the cell division cycle and wherein the mutation is conditional lethal mutation. A conditional lethal mutation, as defined herein, is a mutant strain whose cells can grow at optimal temperatures but are unable to grow at temperatures either above or below the optimum temperature range. At restrictive temperatures, said mutants display a terminal morphology as they will fail to complete the cell division cycle. Such conditional lethal mutant strains include cdc7, encoding protein kinase essential for G1/S transition (H.J. Yoon et al.,

-16-

Molec. Cell Biol., 4:195 (1983); cdc3, cdc11 and cdc12, involved in bud separation (S.K. Ford et al., Develop. Genet., 12:281 (1991); cdc9 encoding DNA ligase I (A.E. Tomkinson et al., Biochem., 31:11762 (1992); cdc46 which is involved in DNA replication (Y. Chen et al., Proc. Natl. Acad. Sci. USA, 89:10459 (1992); and cdc43 encoding type I geranylgeranyl transferase (M.L. Mayer et al., J. Biol. Chem., 267:20589 (1992)).

In one embodiment where S. cerevisiae is used in a mixed culture of two different strains, the test strain is a cdc mutant, which exhibits a pink color and the control strain is a white, wild type strain. The assay can also be performed with a pink control strain and a white test strain. To construct this assay, the cdc mutant and a wild-type strain are first independently grown at the permissive temperatures and then shifted to a restrictive temperature. Subsequently, the two strains are mixed, at a ratio that will permit detection of the test strain at a permissive temperature, and cultured in an appropriate medium that will support the growth of both strains. The candidate agent is contacted with the culture and then the culture is incubated at a permissive temperature. If the candidate agent inhibits the desired cell division cycle function, the mixed assay culture is white, when the test strain is pink. If the candidate agent is inactive, the culture will be pink.

In an embodiment in which the aspect of the cell division cycle targeted is the start of cell division of S. cerevisiae, the assay is for agents which affect the activation of a CDC28 protein kinase. This

-17-

activation results from contact with cyclins, wherein the cyclin protein family is encoded by the genes CLN1, CLN2 and CLN3.

An S. cerevisiae mixed culture assay can be
5 constructed to identify biologically active agents to inhibit repair of DNA from damage caused by DNA binding agents, which cause double strand breaks (e.g., Bleomycin or daunorubicin) or to counter the effects of X-rays by using a test strain with a mutant rad52
10 allele, which has an increased sensitivity to X-ray and DNA binding agents. These active agents would therefore possess anticancer properties.

An S. cerevisiae mixed culture assay can also be used for toxicity studies by employing a test strain
15 with a repair-deficient mutant rad3, rad6 or rad9 allele. These genes are important components in the repair of damage incurred to DNA by various mutagens that modify the purines and pyrimidines through processes such as alkylation or oxidative deamination.

20 The present method of mixed culture assay can also be used to evaluate immunosuppressive agents. Some immunosuppressive agents are known to function by binding to the peptidyl-prolyl cis-trans isomerases class of proteins. Of these proteins, the cyclophilins
25 act as the receptors for cyclosporin A, while the rapamycin/FK506 binding protein, hereinafter "FKBP", is the receptor for FK506 and rapamycin. Yeast cells in which the gene encoding FKBP had been deleted are totally refractile to agents with structural similarity
30 to rapamycin and FK506. The resistance is highly specific. Typically, yeast cells are very sensitive to

-18-

rapamycin. A color change mixed culture assay can be constructed to detect agents similar to rapamycin/FK506, wherein the test strain includes an FKBP knock-out.

5 An alternate immunosuppressive agent assay can be constructed with two test strains and a control strain. The first test strain includes an FKBP knock-out mutation, while the second test strain includes an FKBP knock-out mutation and a mutation, of a downstream gene
10 affected by the drug-receptor complex, to make the strain resistant to rapamycin. Assay of a candidate agent with the combination of two test strains, each with different colors, can identify highly specific agents.

15 A mixed culture assay can further be used to identify antibiotics which attack bacterial cell walls. For example, a mixed culture of E. coli can include a test strain with a mutation knocking out the function of the mrcB allele (or ponB allele), which encodes the
20 penicillin binding protein 1B, which is deleted for the lacZ gene encoding β -galactosidase. Because of the mrcB mutation, the test strain is susceptible to some cell-wall targeting agents. The control strain in this assay is a congenic strain carrying the wild type mrcB
25 allele, which also expresses a reporter gene, for example, the lacZ gene encoding β -galactosidase. This reporter gene is expressed constitutively, for example, by including a lesion in the lacI gene. When an indicator compound, such as 5-bromo-4-chloro-3-indolyl-
30 β -D-galactopyranoside (hereinafter "X-gal"), is included in the mixed assay, the control cells are

-19-

colored whereas the test cells are unpigmented. Where "X-gal" is the indicator compound, the control cells will be blue. By mixing the control and test strains in a suitable ratio, the mixed culture will display
5 white color in the absence of cell-wall targeting agents, but will turn blue after exposure to cell-wall targeting agents. The test strain is known to be susceptible to β -lactam antibiotics, which can be used as a control agent to optimize the mixed culture.

10 In another cell-wall targeting agent assay, two unrelated strains are used in a mixed culture. One cell strain is a test strain which includes the targeted cellular component, specifically the cell wall, while the control strain lacks a cell wall, and thus is not
15 susceptible to cell wall targeting agents. Suitable test strains include, Bacillus subtilis. Suitable control strains include Acholeobacter laidlawii. Either strain can express a reporter gene, such as lacZ, which produces a strain with a color when fermented in the
20 presence of an indicator compound.

A mixed culture assay can also be used to evaluate agents which impair the function of essential bacterial targets, such as RNA polymerase which is essential for transcription, the protein secretion system, cell
25 division, ribosomes which carry out protein synthesis, and DNA gyrase which is an essential function in maintaining DNA in its correct conformation. RNA polymerase, ribosomes, and DNA gyrase are proven bacterial targets in that antibiotics have been
30 isolated that attack them.

-20-

Where the targeted cellular component is a ribosome, the mixed culture assay can use a test strain containing a mutation in a ribosomal gene which causes a defect in the ribosome which increases susceptibility to anti-ribosomal agents. For example, mutations resulting in the loss of the rpsT gene of E. coli, encoding ribosomal protein S20 of the 30S ribosomal subunit, causes a reduction of the cell growth rate (F. Gotz et al., "Escherichia Coli 30S Mutants Lacking Protein S20 are Defective in Translation Initiation", BBA 1050:93 (1990); or the loss of the rplX gene of E. coli, encoding the L24 protein of the 50S subunit, also impairs the growth rate of E. coli. The test strain, in this assay can carry either mutated allele, and is deleted for the lacZ gene. The control strain contains the wild type ribosomal genes, and will also carry the lacZ gene which will endow the cell with blue color grown in the presence of X-gal.

In another embodiment, some mutations encoding the target functions are conditional lethal mutations for which the genetic defect is more severe at high temperatures of cultivation. Thus, temperature, as well as ratio of cell types, can be adjusted when a temperature-sensitive allele is used, to obtain the most effective color change in the mixed culture exposed to known inhibitors.

For example, the mixed culture assay can be used to screen for agents that affect the secretion of proteins in bacteria. Conditional lethal mutations in secA, secD, secE, secF, or secY, will result in lethality if cells are incubated at a nonpermissive

-21-

temperature, due to impaired protein secretion (P.J. Schatz and J. Beckwith, Genetic Analysis of Protein Export in Escherichia Coli, Annual Rev. Genet., 24:215 (1990)). The test strain in this assay contains a
5 conditional lethal mutation resulting in impaired secretion, such as a temperature-sensitive secA allele, and is deleted for the lacZ gene. The control strain includes a wild type sec allele, and also expresses the lacZ gene. A temperature will be determined that
10 impairs protein secretion in the test strain. By mixing the control and test strains in a suitable ratio, the mixed culture will display white color in the absence of biologically active agents, but will turn blue after exposure to biologically active agents
15 such as agents that further impair secretion. Where the test strain contains the defective secA allele, azide, whose primary target is the SecA protein, can be utilized to optimize the mixed culture assay.

The assay can also be used to evaluate potential
20 RNA polymerase inhibitors by utilizing mutations in rpoB or rpoC, which exhibit a conditional lethal phenotype. For example, certain mutations in the rpoC gene will result in an enzyme that is nonfunctional at the restrictive temperature and impaired in function at
25 the permissive temperature (J.H. Miller et al., Altered RNA Polymerases Resulting from Temperature-Sensitive Mutations in the rif Region of E. coli Chromosome, RNA Polymerase, 519-538). The test strain will contain a lesion in rpoC, such as XH56, and is deleted for the
30 lacZ gene. The control strain expresses the wild type rpo allele and includes a lacZ gene. A temperature

-22-

will be determined that impairs transcription in the test strain. Rifampicin, which inhibits RNA polymerase, can be utilized to optimize the mixed culture assay.

5 In another mixed culture assay, DNA gyrase inhibitors evaluated by utilizing mutations in *gyrA* or *gyrB* that exhibit a conditional lethal phenotype. For example, a temperature-sensitive mutation in *gyrB*, results in an enzyme that is severely impaired at the
10 restrictive temperature (S.M. Mirkin and Zh. G. Shmerline, DNA Replication and Transcription in a Temperature-Sensitive Mutant of E. coli with a Defective DNA Gyrase B Subunit, Molec. Gen. Genetic, 188:91 (1982)). The test strain includes a lesion in
15 *gyrB*, and is deleted for the *lacZ* gene, while the control strain expresses the wild type *gyr* allele and contains a *lacZ* gene. Gyrase inhibitors can be utilized to optimize the mixed culture assay.

Further, bacterial cell division also provides
20 other targets which can be used in mixed culture assays by employing strains containing mutations in cell division genes. For example, temperature-sensitive mutations which affect cell division include *ftsZ*, *ftsA*, *ftsQ*, *ftsW*, *ftsH*, and *minB* (J. Lutkenhaus,
25 Bacterial cell division, Emerging Targets in Antibacterial and Antifungal Chemotherapy 117-150 (1992)). The test strain expresses a suitable temperature-sensitive mutation, and is deleted for the *lacZ* gene, while the control strain expresses the wild
30 type alleles and includes a *lacZ* gene. A temperature

-23-

will be determined that impairs growth of the test organism due to a clear defect in cell division.

In yet another embodiment, a mixed culture assay to identify candidate herbicides can be established

5 which targets the genes encoding the production of amino acid biosynthetic enzymes, such as those involved in the synthesis of aromatic amino acids, the leucine-isoleucine-valine pathway, or the biosynthesis of glutamine (G.M. Kishore and D.M. Shaw, Amino Acid

10 Biosynthesis Inhibitors as Herbicides, Annual Reviews of Biochemistry, 57:627-663). The assay is constructed by first cloning the genes, encoding the target from the plant organism, into either bacteria such as E. coli, or yeast, such as S. cerevisiae. For example,

15 the glutamine synthetase gene can be cloned into a first E. coli strain which lacks its own glutamine synthetase, due to mutation, and does not display color due to a deletion of the lacZ gene. Thus growth is dependent on the expression of the cloned gene, since

20 glutamine is absent from the growth medium. In this assay, the growth medium used cannot include glutamine. It is preferred that the expression of the cloned gene would be barely sufficient to allow growth of the E. coli strain in the absence of glutamine

25 supplementation. The second strain of the mixed culture, which exhibits a blue color due to expression of β -galactosidase, contains a mutation or mutations that assures the strong overproduction of active, endogenous glutamine synthetase enzyme. In this assay,

30 contact with a candidate agent possessing biological

-24-

activity results in a change of the color of the mixed culture to blue.

In a further embodiment, a highly specific mixed culture assay to identify agents, such as agonists and antagonists of human or insect hormones, can be constructed by utilizing yeast cells with receptors engineered by recombinant DNA technology. This assay is further described in Example 8. The assay cocultivates two yeast strains that differ in color and in their response to agonists and antagonists of a specific hormone.

The yeast pheromone receptor is modified to produce a fusion protein. The part of the yeast receptor that normally interacts with the G-proteins in the cell and triggers the signal to arrest growth at G1 is retained, but the segment of the molecule that is membrane associated and contains the binding site for the yeast pheromone is replaced by the corresponding region from an exogenous receptor, such as a mammalian or insect hormone receptor. The yeast cells, containing the recombinant receptor, will respond to the exogenous ligand in the same manner that wild type cells respond to yeast pheromone, i.e., growth will be arrested. Strains containing recombinant receptors can be used in mixed culture assays to detect agonists and antagonists of an exogenous ligand, such as described in Example 8.

The invention will now be further and specifically described by the following examples.

-25-

ExemplificationExample 1 - *Saccharomyces cerevisiae* Strain Pair Assay

This assay utilized strains carrying a mutation in one of the genes that codes for a protein which takes part in microtubule function and/or assembly. This mutation increases the sensitivity of yeast strains to drugs affecting tubulin function. The assay used two strains with distinguishing color phenotypes. One strain has red pigmentation due to a mutation in the *ade2* locus. The second strain has a white colony, wild type, phenotype.

In addition, the second strain also carried a mutation that increased the strain sensitivity to microtubule disrupting agents.

To establish the white colony strains which were used in the assay, white colony strains carrying various mutations, possibly affecting microtubules, were tested for benomyl sensitivity in colony formation at 30 °C. The mutations were in the *tub1* gene which encodes α -tubulin, the *tub2* gene which encodes β -tubulin, or in the putative microtubule associated protein genes (hereinafter, "cin"), which were identified by screening for chromosome instability. The *tub1* and *tub2* genes are essential for mitotic division. A mutation in the non-essential *tub3* gene of α -tubulin was also tested.

Benomyl is a member of the benzimidazole class of compounds that have been shown to inhibit microtubule-mediated processes in yeast in vivo and in vitro.

Benomyl inhibits the growth of wild type haploid yeast at concentrations between 15 and 25 $\mu\text{g/ml}$. J.

-26-

Kilmartin, Biochem., 20:3629 (1981); N. Neff et al., Cell, 33:21 (1983); J.H. Thomas et al., Genetics, 12:715 (1985); and R.A. Quinlan et al., J. Cell Sci., 46:341 (1980). The results of the benomyl sensitivity

5 tests were as follows:

Table I

	Strain	Mutation	Growth on Benomyl ($\mu\text{g/ml}$)				
			2	5	10	15	20
10	yG0535	Tub ⁺ , Cin ⁺	+	+	+	+	-
	yG0520	tub1-1	+	-	-	-	-
	yG0521	tub1-1	+	-	-	-	-
	yG0524	tub2-403	+	-	-	-	-
	yG0525	tub2-405	+	+	-	-	-
15	yG0529	tub2-216	+	+	+	-	-
	yG0528	tub2-207	+	+	+	+	-
	yG0531	tub3	+	+	+	-	-
	yG0532	cin1	+	-	-	-	-
	yG0533	cin2	+	-	-	-	-
	yG0534	cin4	+	+	+	-	-

20 The strains yG0521, with a mutation in tub1, yG0524, with a mutation in tub2, and yG0532, with a mutation in cin1, were found to be the most sensitive to benomyl. Thus, strains carrying these mutations generally can not grow on media with benomyl

25 concentrations on which Tub⁺ or Cin⁺ strains can grow. Tub⁺ and Cin⁺ strains are inhibited by 15-20 $\mu\text{g/ml}$ of benomyl whereas tub and cin mutations are inhibited by 2-5 $\mu\text{g/ml}$ of benomyl at 30°C.

-27-

A culture of the yG0535 strain, which is Tub⁺, Cin⁺ (wildtype) and is ade2 (red), was then mixed with a culture of the mutant strain yG0521, yG0524 or yG0532, at a defined ratio so that the colony appeared
5 white. The optimum ratio of strain exists when the appearance of the mixed culture colony is white when grown with no benomyl in the medium and exhibits an area of growth with red pigmentation when the medium contains benomyl. The optimum ratio was established by
10 mixing the strain-pairs in different proportions and then growing the mixed culture colonies on "A" medium and on "A" medium containing increasing concentrations of benomyl. The concentration of DMSO in the medium must not exceed 1% v/v as DMSO concentrations of 1% or
15 higher inhibit the growth of *S. cerevisiae*.

The optimum mixed culture ratio was found to be when the cultures of the white colored tub- strains and the ade2 (red) tub⁺ strain were mixed at a ratio of 9 to 1, respectively, and the plated drop contained
20 50,000 yeast cells.

The strains were grown overnight, in a liquid YEPD-rich medium. The cells were diluted and then mixed at the optimum ratio (9:1) to form the desired mixed culture. Drops of 50 μ L of each mixed culture,
25 each containing 50,000 cells, were added to each well of 96 well-plates containing fungal fermentation products in solid "A" medium.

Prior to adding the mixed culture to the well-plates, 200 μ l of warm "A" medium containing 1.5-2% of
30 Bacto-agar, was added to each well. When solidified, the fungal fermentation products were then added. The

-28-

5 fungal fermentation products, which were obtained from Dr. B. Katz, Myco Search Incorporated were extracted in one of two ways. Broth was extracted after lyophilization and 100 mg of the broth extract was transferred to a microfuge tube and resuspended in 0.4 mL of water. Agar and mycelium were extracted with ethyl acetate. The extract and 0.1 mL of ethyl acetate were then sealed tight in a tube and incubated overnight at 37 °C. After extraction, 20 µl of broth suspension or 5 µl of agar or mycelium suspension were then added to the well-plates. The well-plates were then left overnight to permit the diffusion of the extracts into the medium. The well-plates were incubated at 26 °C or 30 °C for 2-5 days.

15 As a result of the antitubulin activity, the sensitive white strain was inhibited and the area of growth on which the strain pairs were spotted became the red color of the first strain due to a change in the ratio of the two strains in the mixture.

20 Example 2 - S. cerevisiae Drug Resistance Assay

One gene of C. albicans was isolated due to its ability to increase the resistance of S. cerevisiae to benomyl and was referred as BenR. It was found that the gene confers resistance to a number other drugs as well. Thus, the BenR gene was renamed CaMDR (Multiple Drug Resistance of C. albicans).

25 We assume that for a tubulin disrupting agent to affect C. albicans, the resistance provided by this gene or similar genes would have to be overcome. In order to identify products which bypass CaMDR, we

-29-

introduced the CaMDR gene into the genome of the benomyl hypersensitive strains, such as tub1, tub2 or cin mutants. The expectations were that benomyl concentrations that affect the strains with no CaMDR, will not affect the isogeneic strains which contain the CaMDR, unless CaMDR is inhibited or bypassed. Thus, an assay exists that permits the detection of agents that inhibit or bypass the C. albicans benomyl resistance function. Description of the screen is shown in Figure 1.

For the integration of CaMDR into the genome of *S. cerevisiae* we constructed an integrative plasmid, pG0146, which contains CaMDR as shown in Figure 2. It was digested at the *Stu*I site of URA3 and introduced into strains bearing mutations in tub1⁻¹, tub2⁻⁴⁰³, cin1, tub1⁻¹, and to a Tub⁺, Cin⁺ strain (Strains yG0641, 642, 643, 645, 644, respectively).

Strains containing CaMDR and congenic strains without the CaMDR were then tested for their growth on media containing benomyl. As shown in Table II, strains carrying CaMDR were more resistant to benomyl than the isogeneic strains with no CaMDR. The transformants, 641, 642, 643 and 644, were found to be uracil prototrophs and were more resistant to benomyl.

The strains were grown overnight, in liquid YEPD, to stationary phase. The next morning strain-pairs were mixed, 90% of the CaMDR cin⁻ or tub⁻ strains with 10% of the tub⁺, cin⁺ ade2 strain. Then the mixed strains were diluted and 50 μ L drops containing 50,000 cells were added to each well. The plates were then incubated at 30°C for 3-5 days.

-30-

Table II

Strain	Relevant Genotype	Growth on Benomyl ($\mu\text{g/ml}$)				
		2	5	10	20	30
521	tub1 ⁻¹	-	-	-	-	-
5 641	tub1 ⁻¹ , CaMDR	+	+	-	-	-
524	tub2 ⁻⁴⁰³	+	-	-	-	-
642	tub2 ⁻⁴⁰³ , CaMDR	+	+	+/-	-	-
532	cin1	-	-	-	-	-
643	cin1, CaMDR	+	+	-	-	-
10 535	Tub ⁺ , Cin ⁺	+	+	+/-	-	-
644	Tub ⁺ , Cin ⁺ , CaMDR	+	+	+	+	+

Note - 1000 cells per drop were plated. Plates were incubated at 30°C for 3 days.

Example 3 - Aspergillus nidulans Strain Pair Assay

15 For the antimicrotubule assay, two alleles of the tubA locus, encoding α -tubulin, were utilized. These were the wildtype tubA⁺ allele and the tubA4 mutant allele, which has been shown to confer hypersensitivity to all tested antimicrotubule agents. Strains carrying

20 either one of these alleles were generated that also possessed wildtype or mutant alleles of the genes responsible for conidial color pigmentation (the wA and yA loci). The central conidial color pathway has been genetically and molecularly characterized. A colorless

25 precursor was converted to a yellow intermediate by the action of the wA product (a polyketide or fatty acid synthase) and this was converted to the mature green pigment by the action of the yA product (a diphenol oxidase). Hence, wA mutant strains produce white

-31-

conidia, yA mutant strains produce yellow conidia and wildtype strains have green conidia.

- The screening test was performed by mixing conidia of two strains such that they possessed different conidial colors and either the tubA⁺ or tubA4 allele. The test compound was applied and the effect scored after the strains have been allowed to grow and conidiate. If the compound has antimicrotubule activity, the tubA4-containing strain was differentially inhibited and there was a deficiency of this strains' conidial color. Various antimicrotubule and antifungal agents were tested with a series of fungal fermentation extracts in which antimicrotubule activity was detected.
- The sensitivity of the various strains to benomyl was tested by inoculating them onto complete medium plates containing various concentrations of this antimicrotubule agent. After 2 days incubation at 37 °C, 50 % inhibition of growth (colony diameter) was obtained at the following benomyl concentrations shown in Table III:

-32-

Table III

	Strains	Relevant Genotype	Concentration ($\mu\text{g/ml}$)
	L0196	tubA4	0.025
	SAA30	tubA4	0.025
5	SAA31	tubA4	0.025
	SAA32	tubA4	0.025
	SAA27	tubA ⁺	0.3
	SAA33	tubA ⁺	0.3
	SAA34	tubA ⁺	0.3
10	SAA35	tubA ⁺	0.3

The viability of the tubA⁺ and tubA4 strains was tested by plating various dilutions of SAA32 and SAA34 conidial stocks onto appropriately supplemented minimal medium (MM) and complete medium (CM) plates.

- 15 After 2 days incubation at 37 °C, the number of viable conidia were determined. The results of the viability test are shown in Table IV.

Table IV

	Strain ^a	Viable Conidia per mL	Viability Difference
20	SAA32 (tubA4)	1.6x10 ⁶ on CM	62.5-fold
		1.2x10 ⁵ on MM	833-fold
	SAA34 (tubA ⁺)	9.0x10 ⁷ on CM	1.1-fold
		7.5x10 ⁷ on MM	1.3-fold

- 25 a - The initial concentration of each strain tested for viability was 1.0x10⁸ conidia/mL. Numerous colonies with aneuploid morphologies were noted for the SAA32 strain.

-33-

Determination of the optimal ratio of tubA4 and tubA⁺ strains was performed by plating various ratios of SAA32 and SAA34, between 10:1 and 500:1, onto complete medium. After 2 days incubation at 37 °C, the conidial color distribution was noted. Optimal mixture of conidial color was obtained at a 100- to 500-fold excess of SAA32. Final ratio of 125:1 and 250:1 were adopted.

Various strains were tested in pairwise combinations to determine which pair of colors shows the best contrast. The strain pairs tested were SAA30(yellow spore)/SAA34(white spore), SAA30(yellow spore)/SAA35(green spore), SAA31(white spore)/SAA33(yellow spore), SAA31(white spore)/SAA35(green spore), SAA32(green spore)/SAA33(yellow spore), and SAA32(green spore)/SAA34(white spore). Strains SAA30, SAA31 and SAA32 are mutant tubA4 strains while SAA33, SAA34 and SAA35 are wild type tubA⁺ strains. In a first color-combination test, conidia from the various strains were mixed in a 1:250 ratio, using 1×10^5 tubA⁺ conidia/plate and 2.5×10^7 tubA4 conidia/plate, and plated onto complete medium. Benomyl at 100 µg/mL was serially diluted (two-fold) and 1 µL of each dilution was applied. Plates were incubated at 37°C for 2 days. Dilutions to 6.25 µg/ml, representing 6.25 ng of benomyl, gave a positive antimicrotubule result which was evidenced by a deficiency of the tubA4 strains' conidial color in the region surrounding the applied agent.

-34-

A second color-combination test was performed on a microtitre tray assay. The test strains were mixed in molten complete medium to final concentrations of 1.5×10^6 conidia/ml for tubA4 strains and 1.2×10^6 conidia/ml for tubA⁺ strains. After loading 50 μ L of this conidia/medium mix onto a 150 μ L underlay of complete medium in each well of the microtitre tray and allowing it to solidify, benomyl at 100 μ g/ml was serially diluted (two-fold) and 2 μ L of each dilution applied. The highest final well concentration of benomyl 1 μ g/mL. The control was solvent containing no benomyl. Trays were incubated at 37 °C for 1.5 days. Dilutions to 1.6 μ g/mL, representing 3.1 ng of benomyl, gave a positive antimicrotubule result as evidenced by a deficiency of the tubA4 strains' conidial color in the region surrounding the applied agent. The conidial color combination which yielded the greatest discrimination, in both color-combination tests, were the green/white combinations.

The specificity of the antimicrotubule assay tested a range of known antimicrotubule agents for activity. The strains used for this test were SAA31, SAA32, SAA34, and SAA35. The antimicrotubule agents used, and their highest final well concentrations, were benomyl (1 μ g/ml), colchicine (10 μ g/ml), vinblastine (100 μ g/ml), vincristine (100 μ g/ml), podophyllotoxin (500 μ g/ml), griseofulvin (1 mg/ml) nocodazol (1 μ g/mL) and taxol (10 μ g/mL). The results of the microtubule disrupting agent activity tests are provided in Table V.

-35-

Table V

<u>AGENT</u>	<u>LOWEST CONCENTRATION WITH ACTIVITY</u>
benomyl	16 ng/ml
colchicine	very weak at 10 μ g/ml
5 vinblastine	no activity at 100 μ g/ml
vincristine	no activity at 100 μ g/ml
podophyllotoxin	500 μ g/ml
griseofulvin	8 μ g/ml
nocodazol	7.8 μ g/ml.
10 taxol	no activity at 10 μ g/ml

The antifungal agents tested, with specificities other than tubulin, were bleomycin, nystatin, oligomycin, hygromycin B, carboxin, and amphotericin B. None of these agents exhibited patterns of inhibition similar to antimicrotubule agents. Nystatin showed an effect opposite to antimicrotubule agents where the tubA4 strains were more resistant than the tubA⁺ strains.

The performance of this assay under actual screening conditions was tested using fungal fermentation products from ten fungal isolates on mixed cultures SAA32/SAA34 and SAA31/SAA35. These fungi were cultured in cornmeal (CM) or YEPD media. Liquid cultures were used to produce the fungal broth and mycelium extracts, while solidified medium cultures were used for agar extracts. Therefore, each fungal isolate was represented by six extracts consisting of the two media types (CM and YEPD) and the three

-36-

extraction types (broth, mycelium and agar). These products were tested by applying 10 μ l of the resuspended broth extracts, 2 μ l of the resuspended mycelium extracts or 2 μ l of the resuspended agar extracts to the appropriate wells of a microtitre tray containing the conidia/medium mixture. Trays were incubated at 37 °C for 1.5 to 2 days.

Extracts from one fungal strain yielded a strong positive antimicrotubule result. In addition, extracts from three other fungi showed weak but consistent antimicrotubule activity. Serial dilutions of these positive extracts yielded a concentration dependent effect.

To test this assay further, one of the fermentation extracts which showed no antimicrotubule activity was doped with different concentrations of benomyl. The sensitivity of this assay was not dampened by the presence of extract as benomyl concentration of 16 ng/ml still showed an effect.

Conidia from the test pair of strains (tubA4:tubA*) were mixed in liquid complete medium to the following final concentrations: 1.5×10^8 : 1.2×10^6 conidia/ml, 1.5×10^7 : 1.2×10^5 conidia/ml, and 1.5×10^6 : 1.2×10^4 conidia/ml. After loading 200 μ L of this conidia/medium mix into each well of the microtitre tray, benomyl at 100 μ g/ml was serially diluted (two-fold) and 2 μ l of each dilution applied. Trays were incubated at 37 °C for 3 days. The results show that the liquid medium assay was approximately two-fold less sensitive than the solid medium assay.

-37-

Example 4 - Mixed culture Assay for Anti-Fungal Chitin
Synthesis Inhibitors

Chitin is a linear polymer of N-acetylglucosamine that is a structural component of the cell walls of fungi and the exoskeletons of arthropods. As shown in Example 6, chitin-deficient strains are substantially less virulent than wild type strains. Therefore, compounds that substantially reduce cell wall chitin, such as by four-fold, are expected to decrease the virulence of C. albicans and are putative therapeutic agents. However, chitin synthesis inhibitors can exhibit low toxicity in mammals because mammalian cells do not contain chitin.

Studies in the yeast S. cerevisiae have shown that there are at least three enzymes that synthesize chitin. Chitin synthase I and chitin synthase II are encoded by the CHS1 and CHS2 genes, respectively, which however make only a small amount (<10%), of the cell wall chitin. Chitin synthase III, however, is required for the synthesis of about 90% of the chitin in a cell, including the chitin in the bud scars and the lateral wall. The genes CSD2, CSD4, and CAL3 are required for chitin synthase III activity. Mutants defective in CSD2, CSD3, CSD4, and CAL3 typically have a four to ten fold reduction in cell wall chitin.

Although the loss of any one of the CHS, CSD, or CAL genes does not inhibit growth, the loss of certain pairs of genes leads to cell death. Specifically, combination of mutations in CHS2 with mutations that cause chitin deficiency (e.g., csd2 and csd4) is lethal.

-38-

This assay utilizes a mixed culture comprising congenic chs2 ade2 (red) and CHS2 ADE2 (white) strains of S. cerevisiae. In the optimized assay, fermentation broths that do not contain a biologically active agent will produce a pink culture, broths that contain an inhibitor that reduces cell wall chitin will produce a white culture, as the red chs2 ade2 strain is selectively killed. Broths that contain a toxin will inhibit the growth of both strains.

10 Example 5 - Mixed culture Assay for Anti-Fungal Chitin-Binding Compounds

The fluorescent dyes Calcofluor, Congo Red, Primulin, and dianethanol bind to chitin. When added to a culture of yeasts, e.g., C. albicans or S. cerevisiae, these dyes inhibit growth. Mutants, which are deficient in chitin, resist fluorescent dye binding and therefore can be used to screen for chitin-binding compounds with antifungal activity. As defined herein, a chitin-deficient mutant is a strain with a sufficient reduction in cell wall chitin. A example of a significant reduction in chitin is a reduction that substantially reduces cell pathogenicity or calcofluor binding. For instance, a 4-fold or greater was found to reduce calcofluor-binding and pathogenically.

25 Fermentation broths will be screened for compounds that inhibit the growth of wild-type cells but do not inhibit the growth of chitin-deficient cells. Various mutants can be isolated and tested for chitin deficiency.

-39-

Congenic *csd2/csd2 ADE2/ADE2* (white, Calcofluor-resistant) and *CSD2/CSD2 ade2/ade2* (pink, Calcofluor-sensitive) strains of *C. albicans* are used in this assay. To determine the optimum ratio of pink and white cells, the two strains are mixed in various proportions and grown in the presence or absence of Calcofluor, the color of the culture is monitored. The construction of those strains is described in Example 7. The optimum ratio of red and white strains is the ratio that produces a pink culture in the absence of Calcofluor, but a white culture in the presence of Calcofluor as growth of the red (*CSD2/CSD2 ade2/ade2*) strain is selectively inhibited by Calcofluor.

The choice of strains for use in this assay is not limited to *C. albicans* *csd2* disruptants. Any chitin-deficient strain could potentially be used, including the *C. albicans* mutants *pcd3*, *pcd4*, *pcd5* or *pcd 9*, as well as the *csd* or *cal* mutants of *S. cerevisiae*.

Example 6 - Reduced-Virulence Chitin-Deficient Mutants of *C. albicans*

Mutants of *C. albicans*, isolated by two different procedures, were evaluated for chitin deficiency and for virulence. Mutants were isolated in the first procedure by treating a *C. albicans* strain ATCC10261 with 3% ethylmethanesulfonate at 30°C in accordance with the method described for *S. cerevisiae* by Sherman et al., Methods in Yeast Genetics 11 (1986). Aliquots of washed cells were plated onto YPD agar (1% yeast extract, 2% peptone, 2% glucose) containing 0.1 mg/ml of Calcofluor. Resistant colonies were obtained after

-40-

3 to 4 days incubation at 30 °C at a frequency of 1 per 10,000 to 100,000 viable cells. For each of the mutants, cell wall chitin was evaluated. Cell wall chitin was measured as described in Bulawa *et al.*, *Cell*, 46:213 (1986) with the exception that *Serratia marcescens* chitinase was replaced by *Streptomyces plicatus* chitinase and cytohellicase was replaced by β -glucuronidase (Sigma G-1512, approx. 600,000 units/g, 2 mg/100 mg wet yeast cells). Both digestions were performed for 2 hours at 37 °C. From a total of fourteen Calcofluor-resistant mutants, four were shown to be chitin-deficient, containing less than 20% of the amount of chitin present in a wild-type cell. The results are provided in Table VI.

15

Table VI

Amount of Chitin in Calcofluor-Resistant
Mutants Obtained by ES Mutagenesis

	Strain	μ g chitin/mg cells	% of wild-type
	ATCC10261	1.73	100
20	pcd2	1.35	71
	pcd3	0.36	19
	pcd4	0.33	17
	pcd5	0.34	18
	pcd9	0.23	12

25

The virulence of chitin-deficient mutants of *C. albicans* ATCC 10261 strains was tested against male ICR mice. The mice were obtained from the Harlan Sprague-Dawley Co., Indianapolis, IN. Male ICR mice, weighing

-41-

between 20-25 grams, were infected by tail vein injection with 10^6 CFU of the C. albicans strains on day zero. Mortality and morbidity were monitored twice daily for a period of 29 days post-infection. Moribund mice were euthanized by cervical dislocation. Kidneys and spleens were removed after death to verify colonization by C. albicans. The virulence study results for mutants of C. albicans ATCC 10261 are provided in Figures 3 and 4.

In the second isolation procedure, disruption of the CaCSD2 gene as described in Example 7, also produced a chitin-deficient, Calcofluor-resistant strain of C. albicans as shown in Table VII.

Table VII

Amount of Chitin in Mutants Homozygous or Heterozygous for a Disrupted Allele of CaCSD2

Strain	Relevant genotype	Chitin (μ g/mg cells)
CACB3B-5	csd2::hisG/csd2::hUh	0.286
CACB8B-5	csd2::hisG/csd2::hUh	0.305
CACB10B-6	csd2::hisG/csd2::hUh	0.291
CACB10B-10	csd2::hisG/csd2::hUh	0.316
CACB3A	csd2::hisG/CSD2	1.34
CACB8B-6	csd2::hUh/CSD2	1.85
CACB10B-8	csd2::hUh/CSD2	1.80
CAI-4	CSD2/CSD2	1.98

Cell wall chitin was measured by the Bulawa method.
 Average, Csd2⁺ = 1.74 μ g/mg cells.
 Average, Csd2⁻ = 0.30 μ g/mg cells or 17% of normal level.

-42-

The mutations in *csd2* are deletion disruptions:
csd2::hUh represents *csd2::hUh* represents
csd2::hisG_URA3_hisG.

The virulence of chitin-deficient *csd2/csd2* and
5 *csd2/CSD2 C. albicans* mutant strains was tested against
male ICR mice. Male ICR mice, weighing between 20-25
grams, were infected with 10^6 CFU of the *C. albicans*
strains on day zero in accordance with the method
described in F.C. Odds, *Candida and Candidosis 2nd*
10 *Ed.*, W.B. Saunders, London 280 (1988) and N. Khadori *et*
al., *Antimicrobiol. Agent Chemother.*, 37:729 (1993).
Mortality and morbidity were monitored twice daily for
a period of 29 days post-infection. Moribund mice were
euthanized by cervical dislocation. Kidneys and
15 spleens were removed after death to verify colonization
by *C. albicans*. The virulence study results for
csd2/csd2 and *csd2/CSD2 C. albicans* strains are
provided in Figure 5.

20 Example 7 - Construction of C. albicans Chitin-
Deficient Mutants by Gene Disruption

The CaCSD2 gene is a homolog of *S. cerevisiae*
CSD2. CaCSD2 has also been designated CHS3. The
nucleotide sequence of CaCSD2 can be found in GenBank
D13454. CaCSD2 was synthesized in two pieces from *C.*
25 *albicans* chromosomal DNA using two pairs of
oligonucleotide primers and the polymerase chain
reaction. The first primer pair was synthesized from a
first oligonucleotide (5'CCCAGGCCTCACACAGATCATTCGC; SEQ
ID NO:1) and a second oligonucleotide
30 (5'GTGAATCACGCTTACCTC; SEQ ID NO:2).

-43-

In the formula for the first oligonucleotide, the CACACAGATCATTCGC nucleotides, corresponding to bases 10-25 of SEQ ID NO:1, are nucleotides 27-43 of GenBank D13454. In addition, a StuI site was added at the 5' end of the first oligonucleotide to facilitate subsequent cloning. The second oligonucleotide, was the complement of the nucleotides 2648-2665 of GenBank D13454. Using polymerase chain reaction and the first primer pair, a 2.6 kb fragment was synthesized. The pairing reaction was carried out in a solution of 10 mM Tris-HCl, (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each of dNTP, approx. 1 µM of each primer, 5-10 ng/µl SGY243 chromosomal DNA, and 0.05 unit/µl TAQ polymerase. After incubation at 92°C for 3 minutes, the mixture was subjected to 31 amplification cycles, comprising 1 minute denaturation at 92°C, 30 seconds annealing at 62°C, and a 3 minute extension at 72°C, followed by a 7 minute incubation at 72°C.

The second primer pair was synthesized from a third oligonucleotide (5'CGATGAAACTGTGCCACCAG; SEQ ID NO:3), which was nucleotides 2547-2566 of GenBank D13454, and a fourth oligonucleotide (5'CCCTCTAGAGGGACCCTTGAGTATTAGC; SEQ ID NO:4) wherein the complement of nucleotides 4551-4570 of GenBank D13454, corresponding to bases 10-28 of SEQ ID NO:4, was GGGACCCTTGAGTATTAGC and an XbaI site was added at the 5' end to facilitate subsequent cloning. Using polymerase chain reaction and the second primer pair, a 2.0 kb fragment was synthesized. The polymerase chain reaction was the same as for the first primer pair except that the concentration of MgCl₂ was 6 mM.

-44-

The two fragments overlapped by 119 nucleotides. A single Asp718-KpnI site (the only one in GenBank D13454) was located in the overlap region, and the fragments were joined at this site to give the intact
5 CaCSD2 gene. Standard molecular cloning protocols and reagents were used. The first fragment was digested with StuI and Asp718, while the second fragment was digested with XbaI and Asp718. These fragments were ligated into the HincII and XbaI sites of
10 pSKAEcoRIEcoRV, a derivative of pSK lacking the EcoRI and EcoRV sites in the polylinker, to give pCHC2-8.

To make a disrupted allele of CaCSD2 (csd2::hisG_URA3_hisG), 1.8 kb or approximately 50% of the open reading frame was replaced with the "URA
15 blaster" cassette, which is a 4 kb molecular construct consisting of functional *C. albicans* URA3 gene flanked by direct repeats of a bacterial hisG gene. pCHC2-8 was digested with EcoRI, the ends were filled in with Klenow fragment, and BglII linkers, 5'd(pCAGATCTG),
20 were added to yield pCHC2-9. The "URA blaster" cassette was inserted into this plasmid. To obtain the cassette, pMB-7 was cut with SalII, the ends were made blunt with Klenow, and then a second digestion was performed with BglII. The 4 kb fragment was ligated
25 into the EcoRV and BglII sites of pCHC2-9 to give pCHC2-11.

The pCHC2-11 was digested with PstI and NotI to produce a 7 kb linear fragment containing the disrupted gene. Approximately 1 µg of this digest was used to
30 transform CAI-4 (ura3::imm434/ura3::imm434). Uridine prototrophs were selected on solid synthetic medium

-45-

(SD; 0.7% Difco yeast nitrogen base, 2% glucose, 2% agar). Several transformants were grown to saturation in medium (YPD) that contains uridine. In a small percentage of the cells, recombination occurred between the hisG repeats, deleting URA3 and one copy of hisG. The URA⁻Cacsd2::hisG/CSD2 heterozygotes were recovered by plating a portion of each culture on medium containing 5-fluoroorotic acid prepared as described by Boche *et al.*, Mol. Gen. Genet., 197:354 (1984), except that uracil was replaced by uridine. The Cacsd2::hisG/CSD2 heterozygotes were subjected to a second round of transformation with the Cacsd2::hisG_URA3_hisG as described above.

Two types transformants were obtained at high frequency; 1) Cacsd2::hisG_URA3_hisG/Cacsd2::hisG, due to integration of the disrupted gene at CSD2, and Cacsd2::hisG_URA3_hisG/CSD2, due to the integration of the disrupted gene at Cacsd2::hisG. To distinguish between them, the transformants were scored for Calcofluor resistance on YPD agar containing 0.5 mg/ml Calcofluor. Cacsd2::hisG_URA3_hisG/Cacsd2::hisG is Calcofluor resistant and Cacsd2::hisG_URA3_hisG/CSD2 is Calcofluor sensitive. The genotypes were confirmed by Southern analysis as described by J. Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, 9.31-9.58 (1989).

-46-

Example 8 - Mixed culture Assay for the Detection of
Hormone Agonists and Antagonists

Mating in S. cerevisiae is regulated by two
pheromones, a and α (I. Herskowitz et al., The
5 Molecular and Cellular Biology of the Yeast
Saccharomyces, 2:583-657; G.F. Sprague, Jr., and J.R.
Thorner, The Molecular and Cellular Biology of the
Yeast Saccharomyces, 2:657-745. Sexual differentiation
of yeast cells is based on the hormone they secrete and
10 the receptors they express on the surface membranes.
Cells that secrete the a-hormone express receptors for
the α -hormone and those cells secreting the α -hormone
express receptors for the a-pheromone. The mating
process is triggered by mutual exchange of the
15 diffusible hormones that leads to the transmission of
intracellular signals through the cell-surface G-
protein coupled receptor. The response mediated by the
G-proteins synchronizes the cell cycle of both mates
and arrests their replication at G1. Once replication
20 is arrested processes involved in cell fusion and
nuclear fusion are initiated. The hormones have been
purified and when applied to a culture of the proper
mating type, having the receptor for the hormone used,
cell replication is arrested once the cells reach the
25 G1 state (the period prior to DNA replication) of the
cell cycle. Cells remain arrested unless confronted
with a compatible mate. The effect is similar to the
application of any inhibitory agent.

The genes STE2 and STE3 encoding the receptors for
30 a- and α - have been cloned and sequenced. (Burkholder
and Hartwell, Nucl. Acids Res., 13:8463 (1985);

-47-

Nakayama et al., EMBO J., 4:2643 (1985); Hagen et al., Proc. Natl. Acad. Sci. USA, 83:1418 (1986). The α -hormone receptor protein consists of 431 amino acids and the a-hormone receptor protein consists of 470
5 amino acids. Part of each polypeptide is important for recognition of the hormone and part is involved in the transmission of the signal to the G proteins.

The action of many extracellular signals such as neurotransmitters, hormones, odorants and light are
10 mediated by cell-surface G-protein coupled receptors similar to the mating developmental program of S. cerevisiae (H.G. Dohlman et al., Biochemistry, 26:2657; L. Stryer, Ann. Rev. Cell Biol., 2:391 (1986). The nucleotide sequence of some of the receptors is known.

15 A highly specific mixed culture assay to identify agents, as agonists and antagonists of human or insect hormones, is established by utilizing yeast cells with receptors engineered by recombinant DNA technology. The assay cocultivates two yeast strains that differ in
20 color and in their response to agonists and antagonists of a specific hormone.

The yeast pheromone receptor is modified into a fused protein in a manner similar to the that described by King et al., Science, 250:121 (1990) for the
25 mammalian β -adrenergic receptor. The part of the yeast receptor that normally interacts with the G-proteins in the cell and triggers the signal to arrest growth at G1 is retained but the segment of the molecule that is membrane associated and contains the binding site for
30 the yeast phenomone is replaced by the corresponding region from an exogenous receptor, such as mammalian or

-48-

insect β -adrenergic receptor. Yeast cells containing the recombinant receptor will respond to adrenalin in the same manner that wild type cells will respond to yeast phenomone, i.e., growth will be arrested. Pink ade2 strains containing the recombinant receptor will be mixed with a white ADE2 strain containing the wild type yeast phenomone receptor in a ratio that produces a predominantly pink culture. If the mixed culture is contacted with adrenalin or adrenalin agonists, the culture will turn white because the pink cells containing the recombinant receptor. Similarly, if the mixed culture is contacted with agonists of the yeast phenomone, the culture will be pink.

When similar tests are performed in the presence of the hormone together with other tested compounds antagonists may be identified by visual test since the color of a mixed culture will remain pink, whereas, the control in the presence of the hormone alone will be white.

20 Strain Genotype Testing

Strains were genotype tested by known means. A. nidulans was genotyped by spot inoculation of conidia from a single colony onto a series of appropriately supplemented minimal medium plates. Each plate was formulated to test the genotype of one particular locus. For example, strain SAA32 had the genotype bia1; galA1; pyroA4; tubA4. This strain was tested by spot inoculation onto the following plates:

30 - MM+PYRO+NO3 This plate lacks biotin and tests for the bia1 allele.

-49-

- MM+BI+N03 This plate lacks pyridoxin and tests for the pyroA4 allele.
- CF+BI+PYRO+GAL+N03 This plate contains galactose as the carbon source and tests for the galA1 allele.
- MM+BI+PYRO+N03+benomyl This plate contains benomyl at a concentration that severely inhibits the tubA4 containing strain but not the tubA⁺ containing strain.
- MM+BI+PYRO+N0, This plate contains all the requirements for SAA32, which should grow on this media. The conidial color of this strain can be examined on this plate and should be green for SAA32.

The spot inoculation test was performed by inserting a sterile inoculation needle into a single colony to sample conidia and then stabbing the needle into the media of each test plate to deposit conidia. Inoculation was performed with the plate in the inverted orientation by bringing the needle up from the bottom. Up to 5 different inoculations can be performed with a single syringe of conidia.

The plates were incubated in the inverted orientation at 37 °C for 2 days, at which point the genotype was assessed. Positive and negative control strains were included on each plate.

Onto the purified and genotyped colony was placed 200 µL of 7% milk solution and then the conidia was suspended in the milk by using a sterile microbiological loop. 100 µL of the conidial

-50-

suspension was aspirated and placed into a vial, containing sterile, dry silica gel, wherein the vial was sculler to about 0 °C. The vial was then resealed tightly. The contents of the vial were then vortexed
5 thoroughly to obtain a homogeneous distribution of the contents. The silica stock was stored at about 4 °C.

The results of the genotype testing were as indicated in Table VIII.

Table VIII

10	<u>Strain</u>	<u>Genotype</u>
	<u>S. cerevisiae strains</u>	
	yGO535	MATa, ura3-52, leu2,3-112, his3Δ200, ade2-101
	yGO521	MATa, tub-1, ura3-52, leu2,3-112, his3Δ200, ade2-101
15	yG0524	MATa, tub2-403, ura3-52, his4-539, lys2-801
	yG0532	MATa, cini::HIS3, ura3-52, his3Δ200, leu2,3-112
	yG0641	GOY521 but ura3::URA3, CaMDR
	yG0642	GOY524 but ura3::URA3, CaMDR
20	yG0643	GOY532 but ura3::URA3, CaMDR
	yG0644	GOY535 but ura3::URA3, CaMDR
	yG0645	GOY611 but ura3::URA3, CaMDR

-51-

A. nidulans strains

L0196	proA1, pabaA4, yA2, adE20; wA2; pyroA4; tubA4
SAA27	biA1; galA1; sB3; nicB8
SAA30	proA1, pabaA4, yA2, biA1; galA1; tubA4
5 SAA31	biA1; wA2; galA1; nicB8; tubA4
SAA32	biA1; galA1; pyroA4; tubA4
SAA33	proA1, pabaA4, yA2, biA1; pyroA4
SAA34	biA1; wA2; pyroA4
SAA35	biA1; galA1; pyroA4
10 FGSC26	biA1

MATERIALS

The S. cerevisiae strains yGO535, yGO521, yGO524, yGO532 were obtained from the collection of Dr. Gerald Fink, Whitehead Institute, but originated from studies of Dr. David Botstein's laboratory in the Biology Department at the Massachusetts Institute of Technology. These development of strains is described in the following publications: J.H. Thomas et al., Genetics, 112:715 (1985); T.C. Huffaker et al., J. Cell Biol., 106:1977 (1988); T. Stearns et al., Genetics, 124:251 (1990); and M.A. Hoyt, Molec. Cell Biol., 10:223 (1990).

All other S. cerevisiae strains described (yGO641, yGO642, yGO643, yGO644) were derived by M. Goldway for Myco Pharmaceuticals, Inc. in consultation and planning with Drs. Oppenheim and Koltin. The strains were derived by directed integration of a DNA sequence from C. albicans into a specific chromosome of strains yGO535, yGO521, yGO524, and yGO532. A plasmid pGO146 was constructed by cloning the 5.7kb BamHI fragment

-52-

containing the BenR/MtxR(CaMDR) sequence from C. albicans, described by Fling et al., Mol. Gen. Genet., 227:318 (1991) into the BamHI site of the yeast integrative vector YIp5 (R. Rothstein, Methods Enzymology, 194:281 (1991)). Strains yG0535, yG0521, yG0524 and yG0532 were transformed with the Li acetate procedure of Ito et al., J. Bacteriol., 153:163 (1983). Targeting was performed by digestion of the plasmid pG0146 with StuI.

10 The A. nidulans strains were constructed as follows. Strain RMS010 contains the following genotype: biA1 Δ argB::trpCAB metGJ veA1 trpC801. (M.A. Stringer et al., Genes and Development, 5:1161 (1991). Strain MSF (obtained from the Fungal Genetic
15 Stock Center, Dept. Microbiology, Univ. of Kansas Medical Center, Kansas City, Kansas 66103) has the following genotype: suA1adE20 yA2 adE20 AcrA1 galA1 pyroA4 facA303 sB3 nicB8 riboB2. Strain FGSC411, also
20 obtained from the Fungal Genetic Stock Center, has the following genotype: proA1 pabaA1 yA2. Strains MSF and RMS010 were crossed to yield strain SAA20 which has the following genotype: biA1 galA1 pyroA4 facA303 nicB8 riboB2. Strain SAA27 was crossed with strain L0196,
25 (B.R. Oakley et al., Molecular and General Genetics, 208:135 (1987). All crosses were done as described previously (A.J. Clutterbuck, Aspergillus Nidulans Genetics, Handbook of Genetics Vol. 1, (1974).

-53-

The nucleotide sequence of the C. albicans CHS3 gene was obtained from the GenBank DNA data base (accession number D13454, Sudoh, M., Nagahashi, S., Arisawa, M., and Takagi, M., Nippon Roche).

- 5 The following descriptions of media correspond to these media described in Examples 1-6.

"A" medium comprises 6.7 g Bacto-yeast nitrogen base without amino acids, 20 g glucose and 6 mg Adenine sulfate in 1 L distilled water. Benomyl containing
10 media is formed by taking an appropriate amount of a benomyl stock solution, comprising 10 mg of benomyl in 1 mL DMSO which is stored at -20°C, and adding the benomyl stock solution to medium, that is warmed to 50°C, while stirring vigorously.

- 15 A liquid YEPD-rich medium is composed of 10 g Bacto-yeast extract, 20 g Bacto-peptone, 20 g glucose, and 1 L distilled water.

Supplement solution comprises 100 mg of nicotinic acid, 250 mg of riboflavin, 200 mg of pantothenic acid,
20 50 mg of pyridoxin, 1 mg of biotin, and 20 mg of para-aminobenzoic acid. This solution is sterilized by autoclaving for 15 minutes and then stored in light proof container due to reactivity of riboflavin.

Vitamin solution comprises 100 mg of nicotinic
25 acid, 100 mg of riboflavin, 200 mg of pantothenic acid, 50 mg of pyridoxin, 1 mg of biotin, 40 mg of para-aminobenzoic acid, 50 mg of thiamine·HCl, and 400 mg of inositol. The solution is sterilized by autoclaving for 15 minutes. The solution is stored in
30 light proof container due to light sensitivity of riboflavin.

-54-

100xTHIO solution comprises 0.63 M sodium thiosulfate dissolved in distilled water and sterilized by autoclaving.

PROLINE solution (PRO) comprises 2.5 mg/mL

- 5 L-proline dissolved in distilled water and sterilized by autoclaving.

BIOTIN solution (BI) comprises 1 g/mL biotin dissolved in distilled water and sterilized by autoclaving.

- 10 PABA solution comprises 40 μ g/mL para-aminobenzoic acid dissolved in distilled water and sterilized by autoclaving.

- Nicotinic acid solution (NIC) comprises 0.1 mg/mL nicotinic acid or nicotinamide, dissolved in distilled
15 water, and sterilized by autoclaving.

Pyridoxin·HCl solution (Pyro) comprises 50 μ g/mL pyridoxin·HCl, dissolved in distilled water and sterilized by autoclaving.

- 20 GLUCOSE solution (GLU) comprises 0.5 M D-glucose, dissolved in distilled water and sterilized by autoclaving.

GALACTOSE solution (GAL) comprises 0.5 M galactose, dissolved in distilled water and sterilized by autoclaving.

- 25 NITRATE solution (N03) comprises 1 M sodium nitrate, dissolved in distilled water and sterilized by autoclaving.

- 7% Milk solution comprises 7 g of Diploma powdered skim milk dissolved in 100 mL of distilled water and
30 sterilized by autoclaving. This solution is stored at 4 °C.

-55-

Tween Solution comprises 50 μ L of Tween 80 in 1 L of distilled water, which is then sterilized by autoclaving.

Trace Element Solution comprises 40 mg

- 5 $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 400 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 g $\text{FePO}_4 \cdot 4\text{H}_2\text{O}$, 600 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 800 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 8 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 L distilled water. The solution is stored at 4°C in the dark.

- The Salt Solution comprises 26 g KCl, 26 g
10 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 76 g KH_2PO_4 , and 50 mL of Trace Element Solution in 1 L of distilled water. This solution is stored at 4 °C after adding 2 mL of chloroform as a preservative.

- Minimal Medium (MM) comprises 10 g D-glucose and
15 20 mL of Salt Solution in 1 L of distilled water, with the pH of the solution adjusted to 6.5 with 1 M NaOH. Media for plates is solidified with 1% (w/v) agar. Supplemented media is produced by adding 10 ml of
20 SUPPLEMENT solution per liter of media or individual supplements as required by the strain. Sterilize by autoclaving for 15 min. Appropriate nitrogen source is added immediately before use.

- Carbon-Free Medium (CF) comprises 20 mL of Salt Solution and 10 mL of 1M sodium nitrate in 900 mL of
25 distilled water, with the pH then adjusted to 6.5 with 1 M NaOH. Media for plates is solidified with 1% (w/v) agar. Supplemented media is produced by adding 10 ml of SUPPLEMENT solution per liter of media or individual supplements as required by the strain. Sterilize by
30 autoclaving for 15 min. Appropriate carbon source is

-56-

added immediately before use thereby bringing the volume up to 1 liter.

Complete Medium (CM) comprises 10 g D-glucose, 2 g peptone, 1.5 g casein hydrolysate, 1 g yeast extract, 5 10 mLs of 1 M sodium nitrate, 20 mLs SALT solution, 10 mLs of VITAMIN solution, and 10 mLs of riboflavin solution (0.25 mg/mL) in 1 L distilled water with the pH then adjusted to 6.5 with 1 M NaOH. Media for plates is solidified with 1% (w/v) agar and sterilized 10 by autoclaving for 15 minutes.

Silica stocks are prepared for long term storage of conidia. The silica stock is prepared by grinding the silica gel in a coffee grinder to obtain an average particle size of about 1-2 mm. The silica gel is then 15 dispensed into small screw cap vials until they are half full. The silica gel is then sterilized by autoclaving for 15 minutes. Subsequently, the silica gel is dried in a 60-80 °C oven, and then immediately sealed in the vials to avoid moisture. The vials can 20 now be stored at room temperature.

The genetic markers present in the *Aspergillus nidulans* strains used for antimicrotubule testing are listed below. A brief description of the marker and how to test for it is as follows:

- 25 - biA1 Mutant allele of the biA locus. Strains carrying this allele will not grow on media that lack biotin.
- pabaA4 Mutant allele of the pabaA locus. Strains carrying this allele will not grow on 30 media that lack para-aminobenzoic acid.

-57-

- pyroA4 Mutant allele of the pyroA locus.
Strains carrying this allele will not grow on media that lack pyridoxin.
- nicB8 Mutant allele of the nicB locus.
5 Strains carrying this allele will not grow on media lacking nicotinic acid or nicotinamide.
- sB3 Mutant allele of the sB locus. Strains carrying this allele will not grow on media that lack sodium thiosulfate.
- galA1 Mutant allele of the galA locus.
10 Strains carrying this allele cannot grow on media containing galactose as a sole carbon source.
- yA Mutant allele of the yA locus. Strains carrying this allele produce yellow colored conidia.
15
- wA Mutant allele of the wA locus. Strains carrying this allele produce white colored conidia.
- tubA4 Mutant allele of the tubA locus.
20 Strains carrying this allele are hypersensitive to antimicrotubule agents.

All genotyping of strains should be performed on appropriately supplemented minimal medium plates.

- 25 The pabA, pyroA and nicB alleles give clear plus or minus phenotypes. That is, a strain tested for any one of these markers will either grow well or not grow at all on appropriately supplemented medium that lacks the test supplement.

-58-

- The *biA* alleles should also give a clear plus or minus phenotype. However, the *biA1* phenotype can be masked on medium lacking biotin if strains are placed too close to each other such that a *biA1* strain can crossfeed from an adjacent *biA⁺* strain.
- The *sB* alleles exhibit more subtle differences in phenotype due to the presence of sulfur compounds in most agars. Both *sB3* and *sB⁺* strains will grow in media lacking sodium thiosulfate, but growth of the *sB3* strain should be weak and sparse.
- The *galA* alleles also exhibit subtle differences in phenotype due to the presence of other carbon sources in most agars. Strains carrying the *galA1* allele should exhibit weaker growth on medium containing galactose as the sole carbon source. The difference is accentuated by extended incubation times (3 days).
- The phenotypes of the *yA* and *WA* alleles should be very clear when these strains are grown on fully supplemented medium. Strains that carry wild type alleles at both of these loci (*yA⁺* and *WA⁺*) will produce green conidia.
- Mutated *tubA* alleles should produce clear difference in growth when the strains are grown on fully supplemented medium to which 0.1 μ g/ml benomyl (final concentration) has been added. Under these conditions, growth of the wildtype (*tubA⁺*) should not be inhibited,

-59-

while that of a tubA4 strain will be severely inhibited.

Equivalents

Those skilled in the art will recognize, or be
5 able to ascertain, using no more than routine
experimentation, many equivalents to specific
embodiments of the invention described specifically
herein. Such equivalents are intended to be
encompassed in the scope of the following claims.

-60-

CLAIMS

1. A method of evaluating the biological activity of a candidate agent against a targeted cellular component, comprising the steps of:
 - 5 a) providing a first cell strain which displays a first color;
 - b) providing a second cell strain which displays a second color, wherein said second cell strain is more sensitive than said first cell strain to a biologically active agent;
 - 10 c) contacting a suitable ratio of the first cell strain and the second cell strain with a candidate agent and a culture medium, wherein said medium is appropriate for growth of said first and second cell strains, thereby forming a mixed culture assay;
 - 15 d) culturing said mixed culture assay for a suitable period of time under conditions appropriate for growth; and
 - 20 e) evaluating the biological activity of said candidate agent by determining the color displayed by the cultured mixed culture assay, wherein if the color displayed is the first color, the candidate agent is a
 - 25 biologically active agent.

-61-

2. A method of Claim 1, wherein:
 - a) said first cell strain is a strain of a first cell type; and
 - b) said second cell strain is a strain of a second cell type.
3. A method of Claim 1, wherein:
 - a) said first cell strain is a first strain of a cell type; and
 - b) said second cell strain is a second strain of said cell type.
4. A method of Claim 3, wherein said targeted cellular component includes a gene product encoded by a gene.
5. A method of Claim 4, further comprising the steps of:
 - a) providing a first strain which displays a first color and expresses a first type allele of said gene; and
 - b) providing a second cell strain which displays a second color and expresses a second type allele of said gene, wherein said second type allele increases the sensitivity of the second cell strain to said biological activity of said agent.

-62-

6. A method of Claim 5, wherein:
 - a) said first type allele is a wild type allele;
and
 - b) said second type allele is mutant allele.
- 5 7. A method of Claim 5, wherein:
 - a) said first type allele is a mutant allele;
and
 - b) said second type allele is a wild type
allele.
- 10 8. A method of Claim 6 wherein said cell is a fungus
cell.
9. A method of Claim 8 wherein said fungus cell is a
yeast cell.
10. A method of Claim 9 wherein said yeast strain is
15 selected from the group consisting of
Saccharomyces, Aspergillus and Candida.
11. A method of Claim 6 wherein said biologically
active agent is an anti-fungal agent.
12. A method of Claim 11 wherein said anti-fungal
20 agent is a microtubule disrupting agent.
13. A method of Claim 7 wherein said biologically
active agent is an anti-fungal activity.

-63-

14. A method of Claim 13 wherein said biologically active agent binds to a cell wall polysaccharide.
15. A method of Claim 14 wherein the cell wall polysaccharide is selected from the group consisting of: chitin, glucan and a carbohydrate chain attached to mannan.
16. A method of Claim 11 wherein said biologically active agent inhibits chitin production.
17. A method of evaluating the biological activity of a candidate agent against a targeted cellular component which includes a gene product encoded by a gene, comprising the steps of:
 - a) providing a first strain of a cell which displays a first color and expresses a wild type allele of said gene;
 - b) providing a second strain of the cell which displays a second color and expresses a mutation of said gene, wherein said mutation increases the sensitivity of the second strain to a biologically active agent;
 - c) contacting a suitable ratio of the first strain and the second strain with a candidate agent and a culture medium, wherein said medium is appropriate for growth of said strains, thereby forming a mixed culture assay;

-64-

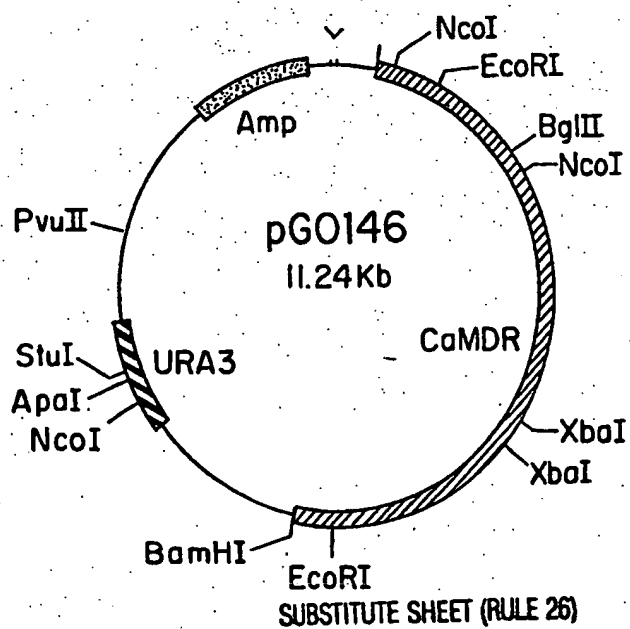
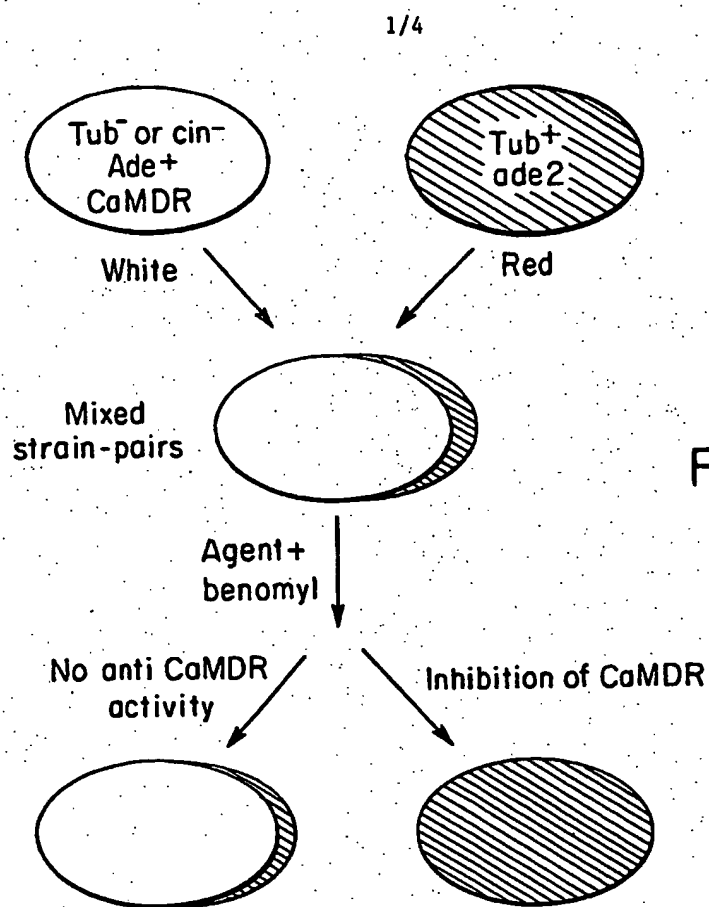
- d) culturing said mixed culture assay for a suitable period of time under conditions appropriate for growth; and
- e) evaluating the biological activity of said candidate agent by determining the color displayed by the mixed culture assay cultured in step (d), wherein if:
- i) the color of said culture is substantially the second color, the candidate agent is not a biologically active compound;
 - ii) the color of said culture is the first color, the candidate agent is a specifically active biologically active compound; and
 - iii) said culture is colorless, the candidate agent is generally active biologically active compound.
18. A method of Claim 17 wherein said fungus is a yeast selected from the group consisting of: *Saccharomyces*, *Aspergillus* or *Candidia*.
19. A method of Claim 2 wherein said first and second cell types are different bacteria cells.
20. A method of evaluating the biological activity of a candidate antibiotic against a targeted cellular component which includes a gene product encoded by a gene, comprising the steps of:

-65-

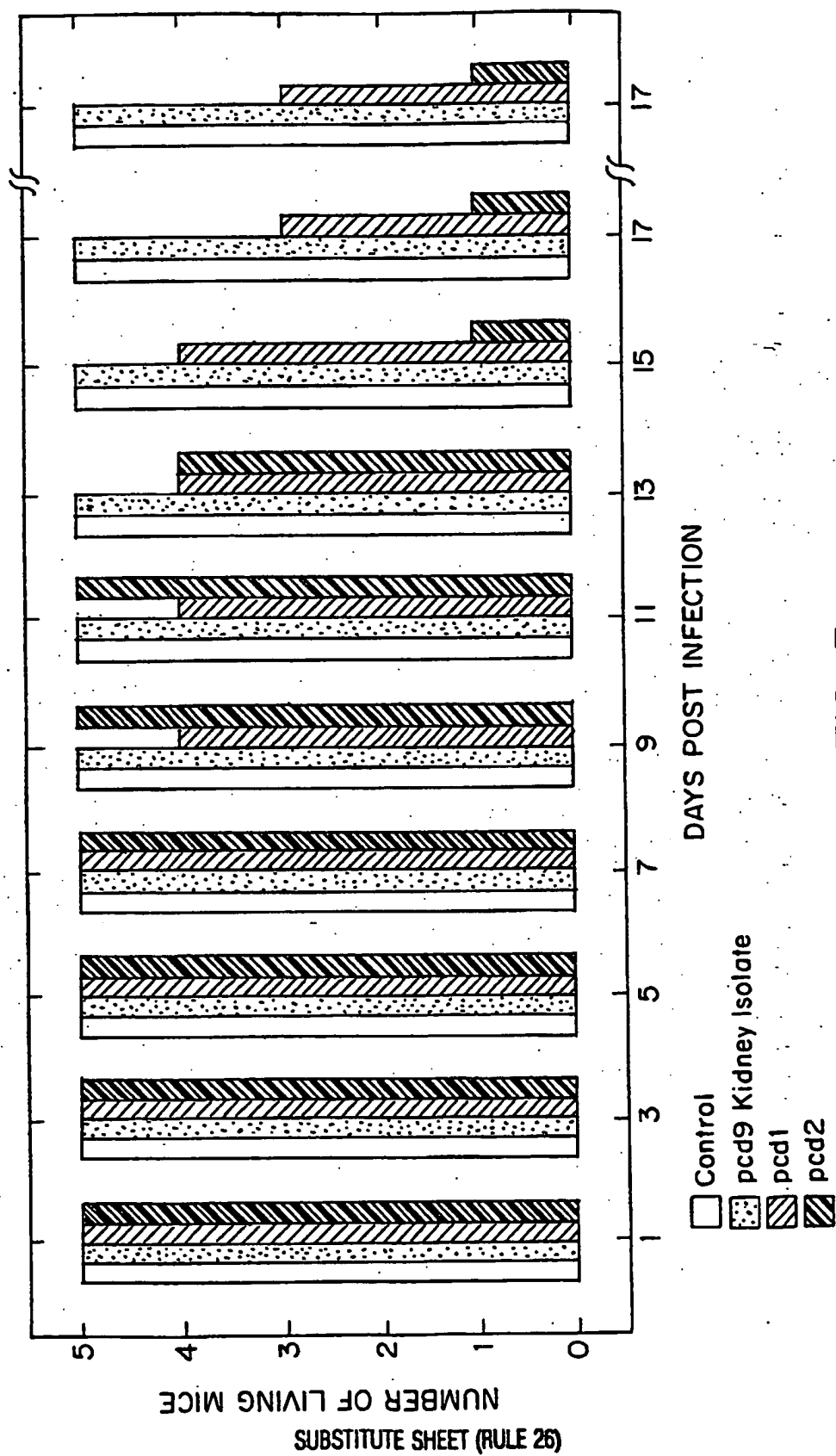
- 5 a) providing a first strain of a bacteria cell which expresses a reporter gene, which produces a colored bacteria cell when contacted with an indicator compound, and also expresses a first type allele of said gene;
- 10 b) providing a second colorless strain of a bacteria cell which expresses a second type allele of said gene, wherein said second strain possesses a sensitivity to a biologically active antibiotic which is different from the sensitivity of said first strain;
- 15 c) forming a mixture of a suitable ratio of the first strain and the second strain, the antibiotic, an indicator compound, and a medium which is appropriate for growth of said strains; thereby forming a mixed culture;
- 20 d) exposing said mixed culture for a suitable period of time to conditions sufficient for bacteria cell growth; and
- 25 e) evaluating the biological activity of said agent by determining if the grown mixed culture is colored.

21. A strain of Candida albicans, wherein said strain is a chitin-deficient mutant strain.

22. A strain of Claim 21, wherein said strain expresses a mutant allele of GenBank D13454.



2/4



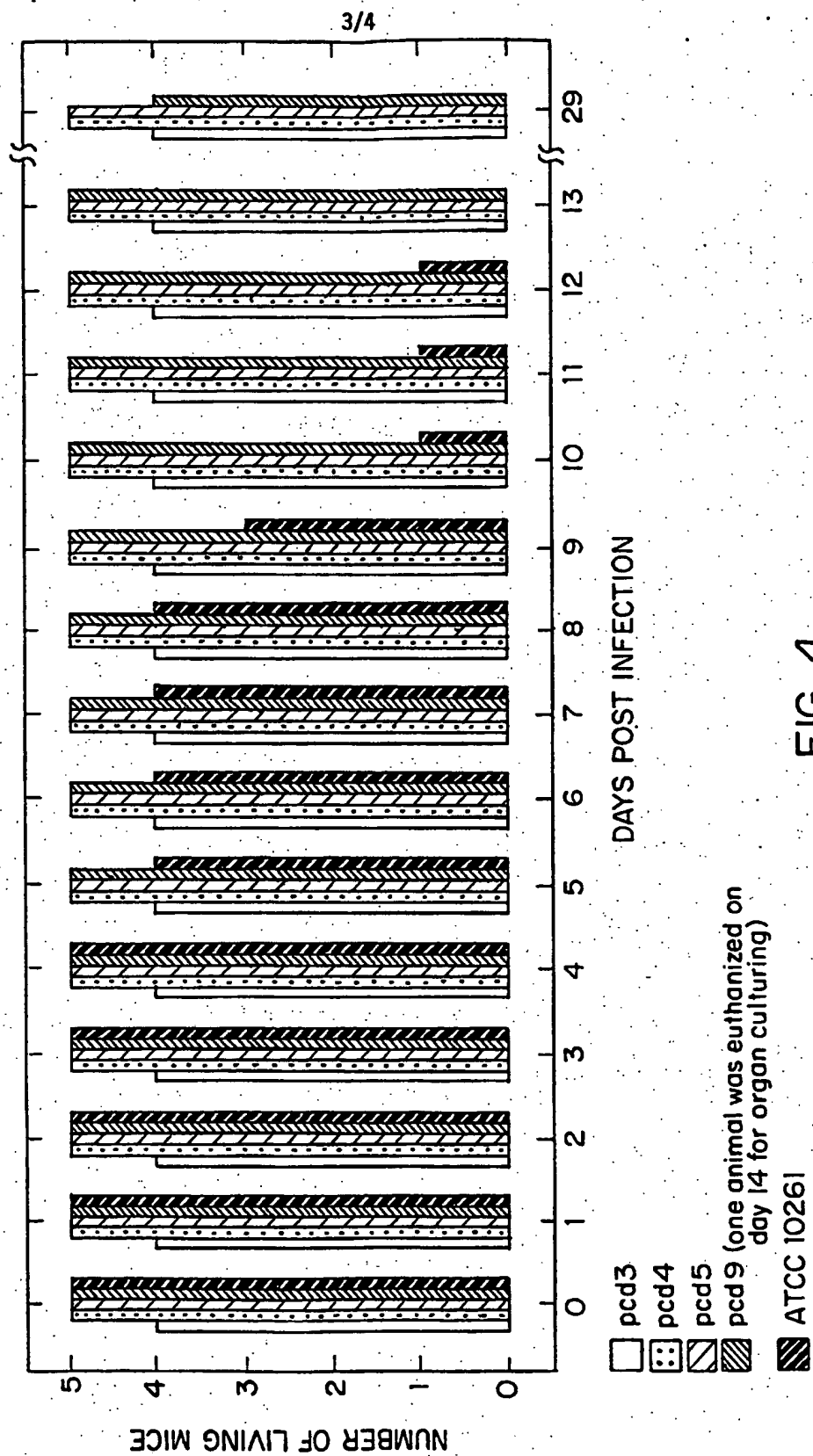


FIG. 4

4/4

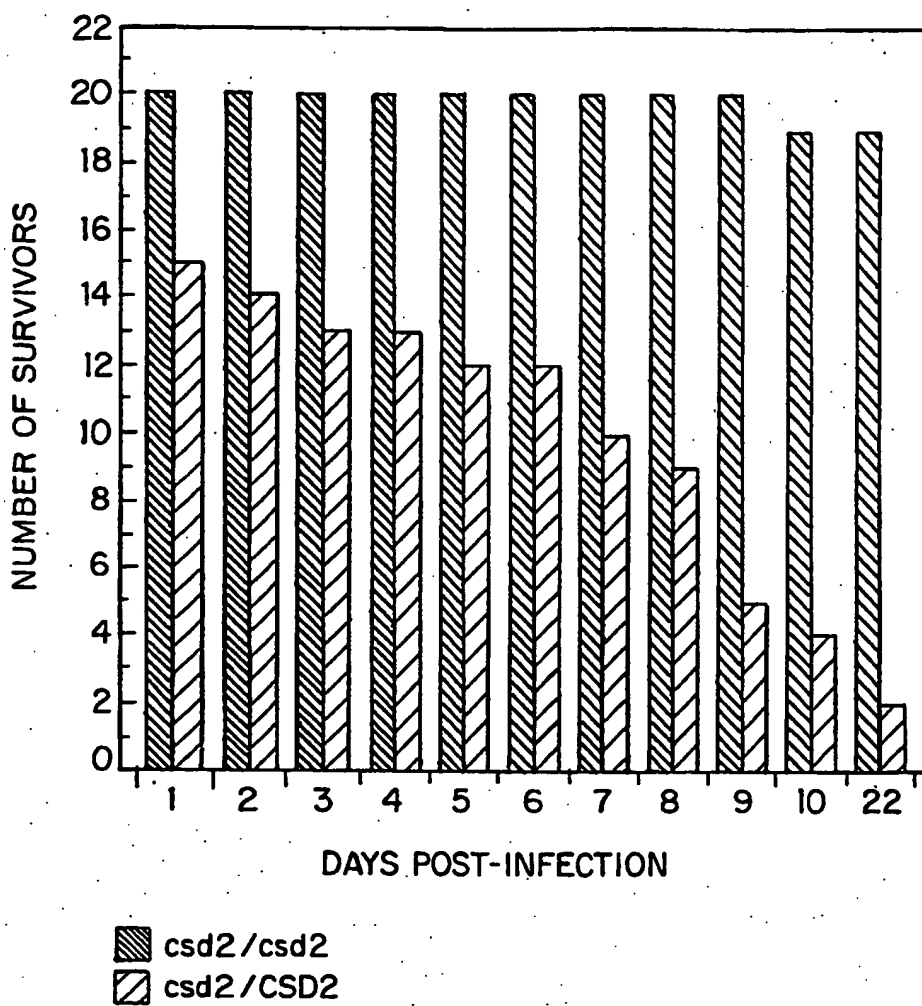


FIG. 5

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(51) International Patent Classification ⁶: C12Q 1/02, 1/18, 1/68, C12N 1/16, 1/19	A3	(11) International Publication Number: WO 95/06132 (43) International Publication Date: 2 March 1995 (02.03.95)
(21) International Application Number: PCT/US94/09304 (22) International Filing Date: 19 August 1994 (19.08.94) (30) Priority Data: 08/113,264 27 August 1993 (27.08.93) US (71) Applicants: MYCO PHARMACEUTICALS, INCORPORATED [US/US]; One Kendall Square, Building 300, Cambridge, MA 02139 (US). UNIVERSITY OF GEORGIA RESEARCH FOUNDATION INCORPORATED [US/US]; Boyd Graduate Studies Research Center, Athens, GA 30602 (US).	(72) Inventors: ANDRIANOPOULOS, Alex; 559 Woodlands Hills Drive, Athens, GA 30606 (US). BULAWA, Christine, E.; 11 Grandview Road, Arlington, MA 02174 (US). GOLDWAY, Martin; 90804 Doar Na Harei Yehuda (IL). KOLTIN, Yigal; 632 Pickering Lane, Phoenixville, PA 19460 (US). MORRIS, N., Ronald; 1488 Main Street, Millstone, Somerville, NJ 08878 (US). O'CONNOR, Sean, Christopher; 88 High Hill Road, Wallingford, CT 06492 (US). OPPENHEIM, Amos; Apartment 12, 5 Harav Shrem Street, 96920 Jerusalem (IL). ROTHSTEIN, David, Michael; 107 Cedar Street, Lexington, MA 02173 (US). TIMBERLAKE, William, E.; 4 Longfellow Place, Boston, MA 02114 (US). (74) Agents: GRANAHAN, Patricia et al; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US). (81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 27 April 1995 (27.04.95)	

(54) Title: IDENTIFYING BIOLOGICALLY ACTIVE AGENTS THROUGH CULTURE COLOR CHANGE**(57) Abstract**

The present invention relates to a method of identifying a biologically active agent, which affects a targeted cellular component, whose existence or function is essential for cell viability, pathogenesis or drug resistance. This assay method includes the use of a mixed culture of first and second cell strains. The cell strains may be either strains of different cells or different strains of the same cell type. The two cell strains are distinguished by color differences. The first cell strain has a first color while the second cell strain displays a second color. The second cell strain is also more sensitive towards a class of biologically active agents than is the first cell strain. The first and second cell strains, and a candidate agent are mixed with a medium, appropriate for growth of the strains, forming a mixed assay culture. The culture is then exposed to conditions appropriate for growth. The initial proportions of the first and second cell strains are such that the culture predominantly favors the development of the second color. The biological activity of the agent is then determined from the color of the culture. Where the mixed culture retains the second color, the agent was not active against the target. However, where the culture predominantly becomes the first color, then the agent showed specific activity towards the target.

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INTERNATIONAL SEARCH REPORT

Inter- national Application No

PCT/US 94/09304

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/02 C12Q1/18 C12Q1/68 C12N1/16 C12N1/19

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 78, no. 25, 25 June 1973 Columbus, Ohio, US; abstract no. 156516, S. NAGAI ET AL. 'Discrimination of the nonfoaming mutant from the parent yeast, Kyokai No. 7, by dyeing method.' page 175; column 1; see abstract & NIPPON JOZO KYOKAI ZASSHI, vol. 67, no.11, 1972 pages 961-963, --- -/--	1-10

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A	CHEMICAL ABSTRACTS, vol. 116, no. 17, 27 April 1992 Columbus, Ohio, US; abstract no. 172667, C. J. JACOBS ET AL. 'Effects of different killer yeasts on wine fermentations.' page 730; column 1; see abstract & AM. J. ENOL. VITIC., vol. 42, no.4, 1991 pages 295-300, ---	
A	BIOLOGICAL ABSTRACTS, vol. 90, no. 2, 15 July 1990 Philadelphia, PA, US; abstract no. 20976, C. GIL ET AL. 'Isolation and characterization of Candida albicans morphological mutants derepressed for the formation of filamentous hypha-type structures.' page AB1119; see abstract & J. BACTERIOL., vol. 172, no.5, 1990 pages 2384-2391, ---	
A	CHEMICAL ABSTRACTS, vol. 107, no. 17, 26 October 1987 Columbus, Ohio, US; abstract no. 151030, H. F. JENKINSON ET AL. 'A mutant of Candida albicans deficient in beta-N-acetylglucosaminidase (chitinase).' page 405; column 1; see abstract & J. GEN. MICROBIOL., vol. 133, no.8, 1987 pages 2097-2106, ---	
E	EP,A,0 626 453 (AMERICAN CYANAMID COMPANY) 30 November 1994 see the whole document -----	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

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